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<p><b>(54) Title</b>  <b>Inducing antibody response against self-proteins with the aid of foreign T-cell eptopes</b></p> <p><b>(51)<sup>6</sup> International Patent Classification(s)</b>  <b>A61K 039/00                      A61K 039/385</b></p> <p><b>(21) Application No: 199870091                      (22) Application Date: 1998.06.11</b></p> <p><b>(30) Priority Data</b></p> <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;"><b>(31) Number</b></td> <td style="width: 33%;"><b>(32) Date</b></td> <td style="width: 33%;"><b>(33) Country</b></td> </tr> <tr> <td><b>964/93</b></td> <td><b>1993.08.26</b></td> <td><b>DK</b></td> </tr> </table> <p><b>(43) Publication Date : 1998.07.30</b>  <b>(43) Publication Journal Date : 1998.07.30</b>  <b>(44) Accepted Journal Date : 1999.07.01</b></p> <p><b>(62) Divisional of:</b>  <b>199476080</b></p> <p><b>(71) Applicant(s)</b>  <b>Mouritsen and Elsner A/S</b></p> <p><b>(72) Inventor(s)</b>  <b>Soren Mouritsen; Henrik Elsner</b></p> <p><b>(74) Agent/Attorney</b>  <b>CARTER SMITH and BEADLE, Qantas House, 2 Railway Parade, CAMBERWELL VIC 3124</b></p> <p><b>(56) Related Art</b>  <b>EP 343460</b>   <b>AU 59581/90</b>   <b>AU 26765/92</b></p>		<b>(31) Number</b>	<b>(32) Date</b>	<b>(33) Country</b>	<b>964/93</b>	<b>1993.08.26</b>	<b>DK</b>
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INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL  
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- (71) Applicant(s)  
MOURITSEN & ELSNER A/S
- (72) Inventor(s)  
SOREN MOURITSEN; HENRIK ELSNER
- (74) Attorney or Agent  
CARTER SMITH & BEADLE , Qantas House, 2 Railway Parade, CAMBERWELL VIC 3124
- (57)

A novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins consists in inserting one or more foreign T-cell epitopes in such proteins by molecular biological means, thereby rendering said proteins immunogenic. The modulated self-proteins can be used as autovaccines against undesirable proteins in humans or animals, said autovaccine being useful as vaccines against a number of diseases, e.g. cancer, chronic inflammatory diseases, rheumatoid arthritis, inflammatory bowel diseases, allergic symptoms or diabetes mellitus.

**Claim**

1. A method for the modification of self-proteins so as to induce antibody response against the unmodified self-proteins following administration of said modified self-proteins to the host, characterized by providing a self-protein analog by molecular biological means by substitution of one or more peptide fragments of the self-protein by a corresponding number of peptides known to contain immunodominant foreign T-cell epitopes, said substitution being carried out so as to essentially preserve the overall tertiary structure of the original self-protein.

2. A method according to claim 1, wherein said immunodominant foreign T-cell epitopes are inserted so as to preserve flanking regions from the original self-protein on both sides of the T-cell epitope.

4. An autovaccine against undesirable self-proteins in humans or animals, characterized in that it comprises one or more self-protein analogs modified according to any of claims 1-3 and formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A and biodegradable polymers.

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**COMPLETE SPECIFICATION**  
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Name of Applicant: **MOURITSEN & ELSNER A/S**

Actual Inventors: **Søren MOURITSEN and Henrik ELSNER**

Address for service  
in Australia: **CARTER SMITH & BEADLE**  
**2 Railway Parade**  
**Camberwell Victoria 3124**  
**Australia**

Invention Title: **INDUCING ANTIBODY RESPONSE AGAINST SELF-  
PROTEINS WITH THE AID OF FOREIGN T-CELL  
EPITOPES**

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The following statement is a full description of this invention, including the best method of performing it known to us

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INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE  
AID OF FOREIGN T-CELL EPITOPES

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5 Field of the invention

This invention concerns a novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins, the presence of which somehow is unwanted in the individual. These could be proteins which are causing disease and/or other undesirable symptoms or signs of disease. Such proteins are removed by circulating autoantibodies which specifically are induced by vaccination. This invention describes a method for developing such autovaccines.

Background of the invention

Physiologically, the vertebrate immune system serves as a defense mechanism against invasion of the body by infectious objects such as micro-organisms. Foreign proteins are effectively removed via the reticuloendothelial system by highly specific circulating antibodies, and viruses and bacteria are attacked by a complex battery of cellular and humoral mechanisms including antibodies, cytotoxic T lymphocytes, Natural Killer cells, complement etc. The leader of this battle is the T helper ( $T_H$ ) lymphocyte which, in collaboration with the Antigen Presenting Cells (APC), regulate the immune defense via a complex network of cytokines.

$T_H$  lymphocytes recognize protein antigens presented on the surface of the APC. They do not recognize, however, native antigen per se. Instead, they appear to recognize a complex ligand consisting of two components, a "processed" (fragmented) protein antigen (the so-called T

cell epitope) and a Major Histocompatibility Complex class II molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)). This recognition eventually enables the T<sub>H</sub> lymphocyte specifically to help B lymphocytes to produce specific antibodies towards the intact protein antigen (Werdelin et al., supra). A given T cell only recognizes a certain antigen-MHC combination and will not recognize the same or another antigen presented by a gene product of another MHC allele. This phenomenon is called MHC restriction.

Normally the individual's own proteins (the so-called self- or autoproducts) are not attacked by the immune apparatus. The described events thus generally are beneficial to the individual, but in rare cases the process goes wrong, and the immune system turns towards the individual's own components, which may lead to an autoimmune disease.

The presence of some self-proteins is inexpedient in situations where they, in elevated levels, induce disease symptoms. High levels of immunoglobulins of the IgE class are e.g. known to be important for the induction of type I allergy, and tumor necrosis factor I (TNF $\alpha$ ) is known to be able to cause cachexia in cancer patients and patients suffering from other chronic diseases (H.N. Langstein et al., Cancer Res. 51, 2302-2306, 1991). TNF $\alpha$  also plays important roles in the inflammatory process (W.P. Arend et al., Arthritis Rheum. 33, 305-315, 1990). Hormones in sex-hormone dependent cancer are other examples of proteins which are unwanted in certain situations. There is therefore a need for the provision of a method for the development of autovaccines against such self-proteins.

Fragments of self-proteins are also presented by the APC, but normally such fragments are ignored or not recognized by the T helper lymphocytes. This is the main reason why individuals generally do not harbor autoantibodies in their serum.

It is, however, possible artificially to induce antibodies against self-proteins. This can be done, as previously mentioned, by covalent conjugation of the self-protein to an appropriate large foreign carrier protein as e.g. tetanus toxoid or key-hole limpet hemocyanin (KLH). Talwar et al. (G.P. Talwar et al, Int. J. Immunopharmacol. 14, 511-514, 1992) have been able to prevent reproduction in women using a vaccine consisting of a conjugate of human chorionic gonadotropin and tetanus toxoid. There are also other examples of such autoimmunogenic conjugates which have been used therapeutically in man and in animal models (D.R. Stanworth et al., Lancet 336, 1279-1281 (1990)). During the processing of such conjugates in the APC, the necessary T<sub>H</sub> lymphocyte stimulatory epitopes are provided from the foreign protein eventually leading to the induction of antibodies against the self-protein as well as against the carrier protein. One disadvantage of using this principle is, however, that the antibody response towards the self-protein will be limited due to shielding of epitopes by the covalently linked carrier protein. Another disadvantage is the increased risk of inducing allergic side-effects due to the contemporary induction of an undesired very strong antibody response against the foreign carrier protein.

Other researchers have conjugated a single peptide predicted to be a T cell epitope chemically as a carrier to a self-peptide [D-Lys<sup>6</sup>]GnRH which is a decapeptide acting as a hapten and managed to induce an autoantibody response with MHC restriction to that particular T cell

epitope (S. Sad et al., Immunology 76, 599-603, 1992). This method seems to be more effective compared with conjugation to large carrier proteins. However, it will only induce antibodies in a population expressing the appropriate MHC molecules. This means that a rather large number of different T cell epitopes has to be conjugated to the self-peptide which will eventually disturb the B cell epitopes on the surface of the self-peptide. Extensive conjugation of proteins may furthermore have the opposite effect with regard to immunogenicity (international patent application No. WO 87/00056) and the surface exposed peptide T cell epitopes may be destroyed by proteolytic enzymes during antigen processing (S. Mouritsen, Scand. J. Immunol. 30, 723, 1989), making that method inexpedient. Also, the exact structure of such multi-conjugated self-peptides will not be chemically and pharmaceutically well-defined.

Recently an improved method has been proposed for breaking the B cell autotolerance by chemical conjugation of B and optionally also peptide T cell epitopes to a high molecular weight dextran molecule (WO 93/23076 published November 25, 1993). The disadvantages mentioned above, however, also hold true for said method.

It has been suggested previously that a universally recognized strong T cell epitope could be associated with a foreign peptide having an antigenic structure representing a B-cell epitope using recombinant DNA technology (EP-A2-0 343 460) It has also been suggested to use peptidyl resin conjugates comprising an immunogenic or antigenic peptide incorporating a helper T-cell ( $T_H$  lymphocyte) reactive epitope and preferably a B-cell reactive epitope in the preparation of immunogenic compositions, e.g. vaccines. The conjugates are prepared by solid phase synthesis, preferably on a polyamide resin. (WO



90/15627). While the intent is to increase an antibody response towards the peptides in question, it has not been proposed that this could be done with the purpose of breaking the autotolerance of the immune system and induce an antibody response against self-proteins. Using these methods for induction of autoantibodies against self-proteins one a priori would expect the same rules to be true with regard to the above-mentioned limitations of the MHC restriction of the response. Surprisingly, however, by modulation of self-proteins using the method according to the invention, wherein a self-protein analog is produced by substitution of one or more peptide fragments by a corresponding number of peptides known to contain immunodominant T-cell epitopes, said substitution being carried out so as to essentially preserve the overall tertiary structure of the original self-protein, it proved possible to induce an equally fast and even a stronger autoantibody response against TNF $\alpha$  despite the fact that the inserted T cell epitope used was not restricted to the MHC molecules of the immunized mice, vide Example 3 below. The reason for this observation is not clear but may be due to the appearance of new MHC binding segments in the mutagenized area in the self-protein. However, the experiment shown in example 5 demonstrates that this is probably not the case, since synthetic peptides representing overlapping regions of the implanted ovalbumin T cell epitope in ubiquitin did not bind strongly to any of the MHC class II molecules of the H-2<sup>k</sup> mice in which this recombinant molecule was highly immunogenic.

Most of the potential MHC class II binding segments of a protein are normally cryptic and will not be presented to the host T cells by the antigen presenting cells (S. Mouritsen et al, Scand. J. Immunol. 34, 421, 1991). The observed lacking correspondence between the MHC restric-

tion of the inserted T cell epitope and the restriction of the antibody response could perhaps be due to a general disturbance of the intra-molecular competition of binding to MHC molecules by different self-protein segments. According to the method of the invention non-tolerized cryptic self-protein segments may be presented to the T cells leading to breaking of the T cell as well as the B cell autotolerance towards the protein. In accordance with the invention and illustrated in all the examples described below, a fragment of the self-protein was substituted with a foreign T cell epitope. This deletion followed by a substitution with an other protein fragment minimally obscure the tertiary structure of the self-proteins, but may also contribute strongly to the disturbance of said intramolecular competition of the MHC class II binding self-segments. This concept is therefore clearly different from the above-mentioned prior art mechanisms and methods. Independently of the operating mechanism of action by the method according to the invention, it is more technically advantageous compared to the known methods for breaking the B cell autotolerance, since it is possible to induce antibodies in a broad population of MHC molecules by insertion of a minimal number of different foreign T cell epitopes.

The present invention thus is based on the surprising fact that injection of recombinant self-proteins, which have been appropriately modulated by deletion of one or more peptide fragments and simultaneous insertion of a corresponding number of foreign T cell epitopes, so as to produce a self-protein analog with an essentially preserved tertiary structure, induces a profound autoantibody response against the unmodified self-proteins. Surprisingly the MHC-restriction of the auto-antibody response induced was not necessarily confined to that of the inserted T cell epitope. By inducing minimal tertiary

structural changes in the highly conserved self-protein ubiquitin, as well as in TNF $\alpha$ , foreign T cell epitopes having a length of 12-15 amino acids were inserted using genetic engineering methods. These recombinant self-protein analogs were purified, emulsified in adjuvant and injected into mice. Within only one week an autoantibody response against ubiquitin could be detected in serum from these mice. Non-modified, recombinant ubiquitin treated and injected in the same way was not able to induce a response.

By using this principle for developing vaccines against undesirable proteins, the risk of inducing allergic side-effect is reduced, and toxic self-proteins such as TNF $\alpha$  can simultaneously be detoxified by removing or mutating biologically active protein segments. The epitope-shielding effect described above is not a problem, and autoantibodies against ubiquitin were induced much faster as compared to the known technique, in which the self-protein is conjugated to a carrier protein or peptide. Importantly, by this method it furthermore seems possible to temporarily break the autotolerance of the T cells as well as that of the B cells of the individual, and such recombinant proteins will be self-immunogenic in a large population expressing many different MHC class II molecules.

The vaccine according to the invention consists of one or more self-protein analogs modulated as described above and formulated with suitable adjuvants, such as calcium phosphate, saponin, quil A or biodegradable polymers. The modulated self-protein analogs may be prepared as fusion proteins with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

Accordingly, in one aspect the invention provides a method for preparing a modified self-protein, said self-protein being derived from a vertebrate, the method comprising substituting, by molecular biological means, one or more peptides in the self-protein with a corresponding number of peptides, wherein

- 5 a) the substituting peptides contain immunodominant T-cell epitopes which are foreign to the vertebrate, and
  - b) the substitution(s) carried out essentially preserve(s) the overall tertiary structure of the unmodified self-protein,
- and thereafter confirming that said modified self-protein induces, in the vertebrate, an
- 10 antibody response against the unmodified self-protein following delivery to the vertebrate of said modified self-protein.

In another aspect, the invention provides an autovaccine against undesirable self-proteins in humans or animals,

- 15 characterised in that it comprises one or more self-proteins modified according to the foregoing method and formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A or biodegradable polymers.

- 20 In another aspect the invention provides a modified self-protein comprising one or more peptide fragments containing immunodominant foreign T-cell epitopes wherein the modified self-protein, when introduced in a vertebrate wherein the unmodified self-protein naturally occurs, induces production of antibodies which cross-react with the self-protein,
- 25 with the proviso that the foreign T-cell epitopes(s) is/are inserted so as to preserve flanking regions from the unmodified self-protein on both sides of said T-cell epitope and that the overall tertiary structure of the modified self-protein as compared with the unmodified self-protein essentially being preserved.



The autovaccine may i.a. be a vaccine against  $\text{TNF}\alpha$  or  $\gamma$ -interferon for the treatment of patients with cachexia, e.g. cancer patients, or a vaccine against IgE for the treatment of patients with allergy. Furthermore, it may  
5 be a vaccine against  $\text{TNF}\alpha$ ,  $\text{TNF}\beta$  or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

The invention is illustrated in the following examples:

10 EXAMPLE 1

Substitution of foreign T cell epitopes into ubiquitin

An overview of this procedure is shown in fig. 1 using  
15 the T cell epitope MP7 as example. The gene sequences representing MP7 (MP7.1-C and MP7.1-NC) were synthesized as two complementary oligonucleotides designed with appropriate restriction enzyme cloning sites. The amino acid sequence of MP7 is PELFEALQKLFKHAY, Mouritsen et al,  
20 Scand. J. Immunol. 30, 723-730, 1989. The oligonucleotides were synthesized using conventional, automatic solid phase oligonucleotide synthesis and purified using agarose gel electrophoresis and low melting agarose. The desired bands were cut out from the gels, and known quantities of oligonucleotides were mixed, heated to 5C below  
25 their theoretical melting point (usually to approximately 65C) for 1-2 hours, and slowly cooled to 37C. At this temperature the hybridized oligonucleotides were ligated to the vector fragments containing part of the ubiquitin  
30 gene. The subsequent analysis of positive clones using restriction fragment analysis and DNA sequencing was done by conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2 ed. CSH Laboratory Press, 1989).

EXAMPLE 2Induction of autoantibodies against ubiquitin by vaccination with modified ubiquitin analogs

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Genes containing sequences encoding the foreign T cell epitopes OVA (325-336) from ovalbumin (OVA) and HEL (50-61) from hen eggwhite lysozyme (HEL), respectively, were expressed in E. coli strain, AR58 under control of the heat sensitive S repressor regulated promotor. Expression of the recombinant ubiquitin proteins were verified using a polyclonal anti-ubiquitin antibody and Western-blotting ("Antibodies", Eds.: D. Harlow et al., CSH Laboratory Press, 1988). The recombinant proteins were purified using conventional methods (Maniatis et al., supra).

Mice were injected i.p. with 100 µg of ubiquitin or its analogs in phosphate buffered saline (PBS) emulsified in Freund's Complete adjuvant. Booster injections of the same amount of antigen emulsified 1:1 in Freund's Incomplete adjuvant were performed i.p. at days 14 and 28. Five Balb/c mice in each group were examined and blood samples were examined for the presence of anti-ubiquitin antibodies on day 7, 14, 21, 28, 35, and 42 using conventional ELISA methodology.

The results exemplified by the antibody response against two different ubiquitin analogs containing the T cell epitopes OVA(325-336) and HEL(50-61), respectively, are shown in fig. 2. The amino acid sequence of the inserted OVA(325-336) epitope is: QAVHAAHAEINE and the amino acid sequence of the HEL(50-61) epitope is STDYGILQINSR.

A clear antibody response against native ubiquitin could be detected within only one week from the first injection of antigen reaching a maximum within 2 weeks. Anti-

ubiquitin antibodies produced in rabbits by covalently conjugating ubiquitin to bovine immunoglobulin reached maximum values after a much longer immunization period (data not shown).

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### EXAMPLE 3

#### Induction of autoantibodies against tumor necrosis factors (TNF) by vaccination with appropriately modified TNF analogs

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The gene coding for the structural part of the native murine TNF $\alpha$  protein (MR101) was obtained by Polymerase Chain Reaction (PCR) cloning of the DNA. In the MR103 TNF $\alpha$  analog the ovalbumin (OVA) H-2<sup>d</sup> restricted T cell epitope sequence 325-334 (QAVHAAHAET) replaces the amino acids 26-35 in the cloned TNF $\alpha$  sequence, a substitution of an amphiphatic  $\alpha$ -helix. Substitutions in this region of the TNF $\alpha$  detoxifies the recombinant protein (X. Van Ostade et al., Nature 361, 266-269, 1993). In the MR105 analog the H-2<sup>k</sup> restricted T cell epitope from hen eggwhite lysozyme (HEL), amino acid sequence 81-96 (SALLSSDITASVNCAK) replaces the amino acids 5-20 in the cloned TNF $\alpha$  sequence. In the MR106 TNF $\alpha$  mutant the same epitope, amino acid sequence 81-95 (SALLSSDITASVNCA) replaces the amino acids 126-140 in the cloned TNF $\alpha$  sequence. The genetic constructions are shown in Fig. 3. Different techniques compared to the technique described in example 1 were used for exchanging parts of the TNF $\alpha$  gene with DNA coding for T cell epitopes. The MR105 and 106 constructs were made by introducing the mutant sequence by PCR recloning a part of the TNF $\alpha$  gene flanking the intended site for introducing the T cell epitope. The mutant oligonucleotide primer contained both a DNA sequence homologous to the TNF $\alpha$  DNA sequence as well as a DNA sequence encoding the T cell epitope. The PCR re-

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cloned part of the TNF $\alpha$  gene was subsequently cut with appropriate restriction enzymes and cloned into the MR101 gene. The MR103 construction was made by a modification of the "splicing by overlap extension" PCR technique (R. M. Horton et al., Gene 77, 61, 1989). Here two PCR products are produced, each covering a part of the TNF $\alpha$  gene, and additionally each PCR product contains half of the T cell epitope sequence. The complete mutant TNF $\alpha$  gene was subsequently made by combining the two PCR products in a second PCR. Finally, the complete genetic constructions were inserted into protein expression vectors. Subsequently, all genetic constructions were analyzed by restriction fragment analysis and DNA sequencing using conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2.ed. CSH Laboratory Press, 1989). The recombinant proteins were expressed in E.coli and purified by conventional protein purification methods.

Groups of BALB/c (MHC haplotype H-2<sup>d</sup>) and C3H (MHC haplotype H-2<sup>k</sup>) mice, respectively, were immunized subcutaneously with 100 Tg of semi-purified MR103 and MR106 emulsified in Freund's complete adjuvant. Every second week the immunizations were repeated using incomplete Freund's adjuvant. All mice developed an early and strong antibody response against biologically active MR101. This was measured by a direct ELISA method using passively adsorbed 100% pure MR101 (Fig. 4). Control mice immunized with MR101 and PBS, respectively, showed no antibody reactivity towards MR101.

Strikingly, the antibody response towards MR101 was not MHC restricted corresponding to the implanted T cell epitopes, since both mice strains of different MHC haplotypes responded well to MR103 and MR106 containing differently restricted T-cell epitopes (Fig. 4). Taken together these results illustrate (a) the ability of the



self-protein analogs produced by the method according to the invention to induce autoantibodies towards a secreted autoprotein and (b) the improved efficiency of the herein described method with regard to inducing a response in a broader MHC population than predicted by the MHC binding ability of the inserted T cell epitopes. The immune response against MR101 induced by the recombinant self-protein analogs MR103 and MR106 was much stronger and more high-titered compared to the immune response induced by aldehyde conjugated MR101 (see Example 4).

#### EXAMPLE 4

Induction of autoantibodies against TNF $\alpha$  by self-protein analogs produced by the method according to the invention compared to unmodified self-protein conjugated to E. coli proteins.

The induction of autoantibodies against TNF $\alpha$  by the method of the present invention has been directly compared to the autoantibody response induced when using a conjugate of TNF $\alpha$  and E. coli proteins, which must contain small single T cell epitope peptides as well as larger foreign carrier proteins.

Semi-purified recombinant murine TNF $\alpha$  (MR101) was conjugated to E. coli proteins in PBS, pH 7.4, using 0.5% formaldehyde. Conjugation of the proteins was confirmed by SDS-PAGE. These conjugates were subsequently used for immunization of C3H mice. Another group of C3H mice was vaccinated with semi-purified non-conjugated self-protein analog MR105. About 100  $\mu$ g of recombinant TNF $\alpha$  analog and conjugate were emulsified 1:1 in Freund's complete adjuvant and injected subcutaneously in each group of mice. MR105 is biologically inactive as judged by the L929 bioassay for TNF $\alpha$ . In subsequent immunizations every second

week incomplete Freund's adjuvant was used. Both groups eventually developed autoantibodies against highly purified biologically active MR101 as determined by ELISA, but the immune response against the non-conjugated analog MR105 produced by the method of the invention was induced earlier and was of a higher titer (Fig. 5).

#### EXAMPLE 5

10 The possible MHC class II binding of peptides representing overlapping sequences of self-protein as well as of the ovalbumin T cell epitope inserted in ubiquitin.

Peptide-MHC complexes were obtained by incubating  $^{125}\text{I}$ -labeled peptide (10-100 nM) with affinity purified MHC class II molecules (2-10  $\mu\text{M}$ ) at room temperature for 3 days (S. Mouritsen, J. Immunol. 148, 1438-1444, 1992). The following peptides were used as radiolabeled markers of binding: Hb(64-76)Y which binds strongly to the  $\text{E}^{\text{k}}$  molecule and HEL(46-61)Y which binds strongly to the  $\text{A}^{\text{k}}$  molecule. These complexes were co-incubated with large amounts of cold (non-radiolabeled) peptide (> 550 Tm) which is sufficient to inhibit totally all immunologically relevant MHC class II binding. Either the same peptides were used, or three different overlapping peptides were used, said peptides representing the flanking regions as well as the entire OVA(325-336) T cell epitope which was substituted into ubiquitin (see Example 2). The three peptides were: TITLEVEPSQAVHAA (U(12-26)), PSQAVHAAHAEINEKE (U(19-34)) and HAEINEKEGIPPDQQ (U(27-41)). The reaction buffer contained 8 mM citrate, 17 mM phosphate, and 0.05% NP-40 (pH 5) and peptide-MHC class II complexes were separated (in duplicate) from free peptide by gel filtration using G25 spun columns. Both the radioactivities of the excluded "void" volume and of the included volume were measured by gamma spectrometry. The

competitive inhibition of maximal binding (in percent) by addition of cold peptide was calculated. The results are shown in Table I.

5 TABLE I.

	Peptide/	Hb(64-76)	HEL(46-61)	U(12-26)	U(19-34)	U(27-41)
	MHC					
10	A <sup>k</sup>	28.6	<u>97.4</u>	35.3	44.6	7.8
	E <sup>k</sup>	<u>92.6</u>	0.0	45.6	12.2	0.0

It can be seen that total inhibition of the binding of the radiolabeled peptides Hb(64-76)Y and HEL(46-61)Y to E<sup>k</sup> and A<sup>k</sup> respectively could only be achieved using cold versions of the same peptides. Although some inhibition of binding was seen by U(12-26) and U(19-34) using these extreme amounts of cold peptide, it is likely that the affinity of these peptides to the H-2<sup>k</sup> MHC class II molecules is very low. Therefore this seems not to be sufficient to explain the strong immunogenicity in the H-2<sup>k</sup> mouse strain of the ubiquitin analog containing the ovalbumin T cell epitope. More likely, other and previously non-tolerized self-epitopes are presented to the T cell in these animals.

EXAMPLE 6

30 Treatment of diabetes or inflammatory disease by vaccination with appropriately modified TNF $\alpha$  analogs

Genes coding for TNF $\alpha$  are modified by insertion of appropriate gene segments coding for T cell epitopes derived from e.g. tetanus toxin or influenza hemagglutinin. Such genes are expressed in appropriate expression vectors in

e.g. *E. coli* or insect cells. The recombinant TNF $\alpha$  proteins were purified using conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2. ed. CSH Laboratory Press, 1989).

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Optionally such recombinant proteins can be coupled to immunologically active cytokines such as GM-CSF or interleukin 2.

- 10 The recombinant proteins can be formulated with appropriate adjuvants and administered as an anti-TNF $\alpha$  vaccine to patients suffering from diseases where TNF $\alpha$  is important for the pathogenesis. The induced anti-TNF $\alpha$  antibodies will thereby affect the diseases.

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One example of said diseases is the chronic inflammatory diseases such as e.g. rheumatoid arthritis where TNF $\alpha$  is believed to play an important role (reviewed in: F.M. Brennan et al., *Br. J. Rheumatol.* 31, 293-298, 1992).

- 20 TNF $\alpha$  is also believed to play an important role in the cachectic conditions seen in cancer and in chronic infectious diseases such as AIDS (reviewed in M. Odeh. *J. Intern. Med.* 228, 549-556, 1990). It is also known that TNF participates in septic shock (reviewed in: B.P. Giroir, *Crit. Care. Med.*, 21, 780-789, 1993). Furthermore, it has been shown that TNF $\alpha$  may play a pathogenetic role in the development of type II diabetes mellitus (CH Lang et al., *Endocrinology* 130, 43-52, 1992).

### 30 LEGENDS TO FIGURES

- Fig. 1. Schematic overview of the cloning strategy used in the construction of a ubiquitin gene with an implanted foreign T cell epitope (MP7). Restriction enzyme digestions, hybridization and ligation procedures are indi-

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cated with arrows. Fragment sizes are shown in parentheses.

Fig. 2. Reactivity toward immobilized bovine ubiquitin in sera from mice immunized with recombinant ubiquitin and analogs containing the implanted T cell epitopes OVA(325-336) and HEL (50-61), respectively. Fig. 2a) sera from Balb/c mice immunized with recombinant ubiquitin containing OVA(325-336). Fig. 2b) sera from Balb/c mice immunized with recombinant ubiquitin containing the T cell epitope HEL(50-61). Fig. 2c) sera from Balb/c mice immunized with recombinant non-modified ubiquitin. Sera (diluted 1:100) were tested in a standard ELISA assay using non-modified bovine ubiquitin immobilized on the solid phase.

Fig. 3. Schematic overview of the cloning strategy used in the construction of the recombinant TNF $\alpha$  mutants. PCR products and restriction enzyme digestions are indicated.

Fig. 4. Induction of TNF $\alpha$  autoantibodies by vaccination of Balb/c or C3H mice with semipurified MR103 and MR106. The antibody titers were measured by ELISA and expressed as arbitrary units (AU) referring to a strong standard anti-serum from one mouse. The plotted values represent a mean titer for 5 animals. Freund's complete adjuvant was used as adjuvant for the first immunization. All subsequent immunizations at 14 days intervals were done with Freund's incomplete adjuvant. Mice immunized in parallel with native MR101 in PBS did not develop detectable TNF $\alpha$  autoantibodies (data not shown). Non-detectable antibody titers were assigned the titer value 1.

Fig. 5. Anti TNF $\alpha$  autoantibodies induced by vaccination with non-conjugated MR105 and MR101 conjugated to E. coli proteins, respectively. C3H mice and Balb/c mice were im-

munized with both preparations. The immunizations, measurements and calculations of mean antibody titers were done as described in example 4.

一、二、三、四、五、六、七、八、九、十、十一、十二、十三、十四、十五、十六、十七、十八、十九、二十、二十一、二十二、二十三、二十四、二十五、二十六、二十七、二十八、二十九、三十、三十一、三十二、三十三、三十四、三十五、三十六、三十七、三十八、三十九、四十、四十一、四十二、四十三、四十四、四十五、四十六、四十七、四十八、四十九、五十、五十一、五十二、五十三、五十四、五十五、五十六、五十七、五十八、五十九、六十、六十一、六十二、六十三、六十四、六十五、六十六、六十七、六十八、六十九、七十、七十一、七十二、七十三、七十四、七十五、七十六、七十七、七十八、七十九、八十、八十一、八十二、八十三、八十四、八十五、八十六、八十七、八十八、八十九、九十、九十一、九十二、九十三、九十四、九十五、九十六、九十七、九十八、九十九、一百。

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for preparing a modified self-protein, said self-protein being derived from a vertebrate, the method comprising substituting, by molecular biological means, one or more peptides in the self-protein with a corresponding number of peptides, wherein
  - a) the substituting peptides contain immunodominant T-cell epitopes which are foreign to the vertebrate, and
  - b) the substitution(s) carried out essentially preserve(s) the overall tertiary structure of the unmodified self-protein,
- and thereafter confirming that said modified self-protein induces, in the vertebrate, an antibody response against the unmodified self-protein following delivery to the vertebrate of said modified self-protein.
2. A method according to claim 1, wherein said immunodominant foreign T-cell epitopes are inserted so as to preserve flanking regions from the unmodified self-protein on both sides of the T-cell epitope.
3. A method according to any of claims 1 or 2, wherein the immunodominant T-cell epitope(s) originate(s) from tetanus toxoid or diphtheria toxoid.
4. An autovaccine against undesirable self-proteins in humans or animals, characterised in that it comprises one or more self-proteins modified according to any of claims 1-3 and formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A and biodegradable polymers.
5. An autovaccine according to claim 4, characterised in that the modified self-protein is present in the form of a fusion protein with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.
6. An autovaccine according to claim 4, characterised in that it is a vaccine against TNF $\alpha$  or  $\gamma$ -interferon for the treatment of patients susceptible to cachexia, e.g. cancer patients.
7. An autovaccine according to claim 4, characterised in that it is a vaccine against IgE for the treatment of patients with allergy.



8. An autovaccine according to claim 4, characterised in that it is a vaccine against  $\text{TNF}\alpha$ ,  $\text{TNF}\beta$  or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

5 9. An autovaccine according to claim 8, characterised in that it is a vaccine for treatment of patients with rheumatoid arthritis or an inflammatory bowel disease.

10 10. An autovaccine according to claim 4 or 5, characterised in that it is a vaccine against  $\text{TNF}\alpha$  for the treatment of diabetes mellitus.

11. A method according to any one of claims 1 to 3 substantially as hereinbefore described.

15 12. An autovaccine according to any one of claims 4 to 10 substantially as hereinbefore described.

13. A method of inducing an antibody response against a self-protein, said self-protein being derived from a vertebrate, the method comprising:

20 introducing, in the vertebrate, an effective amount of the self-protein which has been modified by substitution of one or more peptides in the self-protein with a corresponding number of peptides comprising immunodominant T-cell epitopes which are foreign to the vertebrate while preserving the overall tertiary structure of the self-protein.

25 14. Use of a modified self-protein modified according to the method of any one of claims 1-3 for inducing antibodies against the unmodified self-protein in a vertebrate harbouring the self-protein.

30 15. A modified self-protein comprising one or more peptide fragments containing immunodominant foreign T-cell epitopes wherein the modified self-protein, when introduced in a vertebrate wherein the unmodified self-protein naturally occurs, induces production of antibodies which cross-react with the self-protein,

35 with the proviso that the foreign T-cell epitopes(s) is/are inserted so as to preserve flanking regions from the unmodified self-protein on both sides of said T-





cell epitope and that the overall tertiary structure of the modified self-protein as compared with the unmodified self-protein essentially being preserved.

5 16. A modified self-protein according to claim 15 formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A and biodegradable polymers.

10 17. A modified self-protein according to claim 15 in the form of a fusion protein with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

18. A modified self-protein according to claim 15 being a protein for use in the treatment of patients susceptible to cachexia, e.g. cancer patients.

15 19. A modified self-protein according to claim 15 for use in the treatment of patients with allergy.

20 20. A modified self-protein according to claim 15 for use in the treatment of patients with chronic inflammatory diseases.

21. A modified self-protein according to claim 15 for use in the treatment of patients with rheumatoid arthritis or an inflammatory bowel disease.

25 22. A modified self-protein according to claim 15 for use in the treatment of diabetes mellitus.

23. A method according to claim 13 substantially as hereinbefore described.

30 24. A modified self protein according to any one of claims 15 to 22 substantially as hereinbefore described.

DATED: 18 February 1999

**CARTER SMITH & BEADLE**  
Patent Attorneys for the Applicant:

MOURITSEN & ELSNER S/A



# ABSTRACT

A novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins consists in inserting one or more foreign T-cell epitopes in such proteins by molecular biological means, thereby rendering said proteins immunogenic. The modulated self-proteins can be used as autovaccines against undesirable proteins in humans or animals, said autovaccine being useful as vaccines against a number of diseases, e.g. cancer, chronic inflammatory diseases, rheumatoid arthritis, inflammatory bowel diseases, allergic symptoms or diabetes mellitus.

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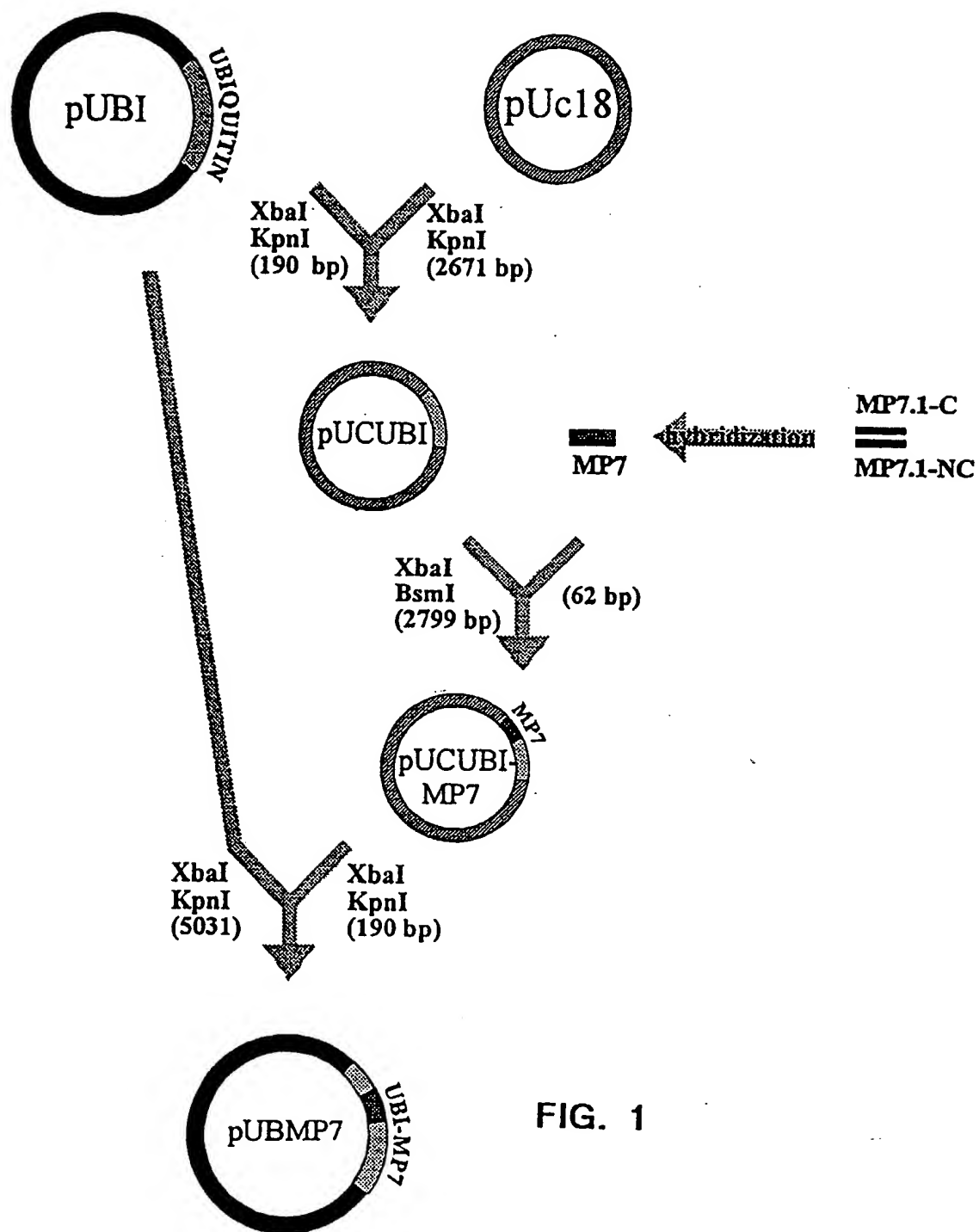


FIG. 1

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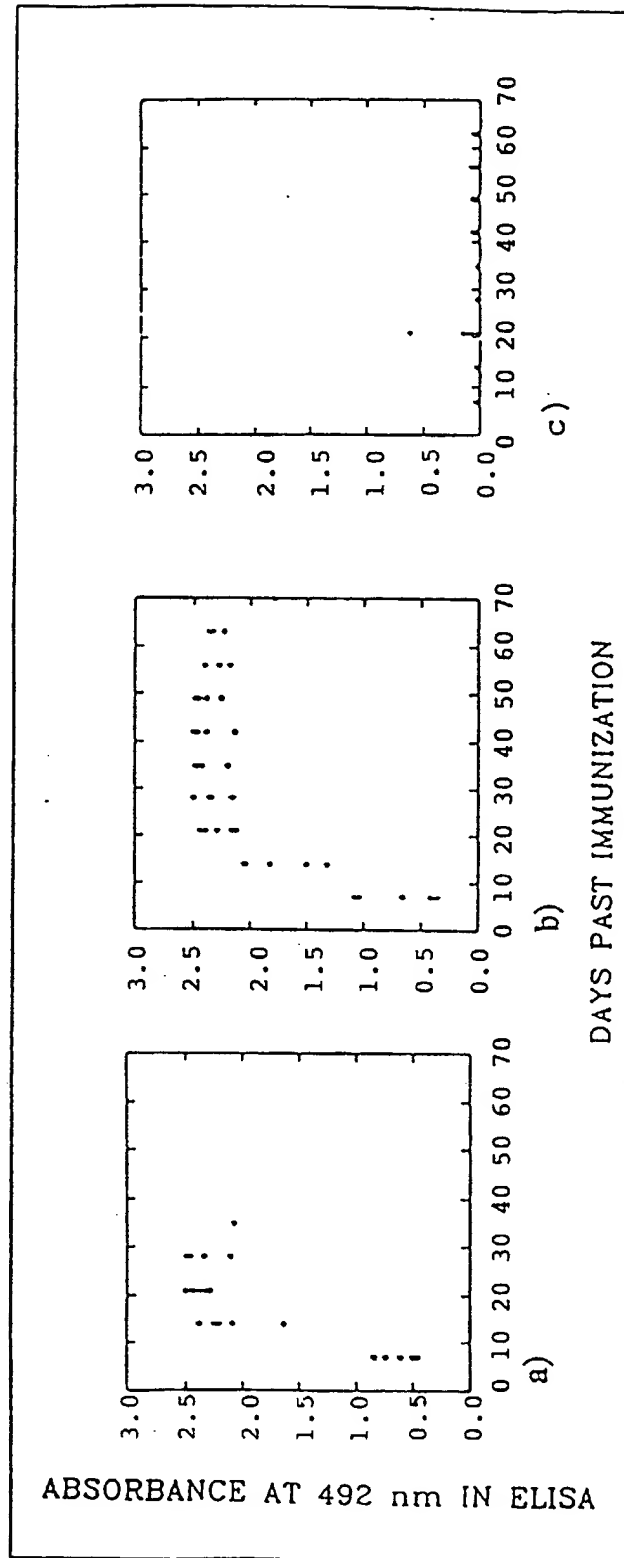


FIG. 2

# Cloning strategy for murine TNF $\alpha$ mutants.

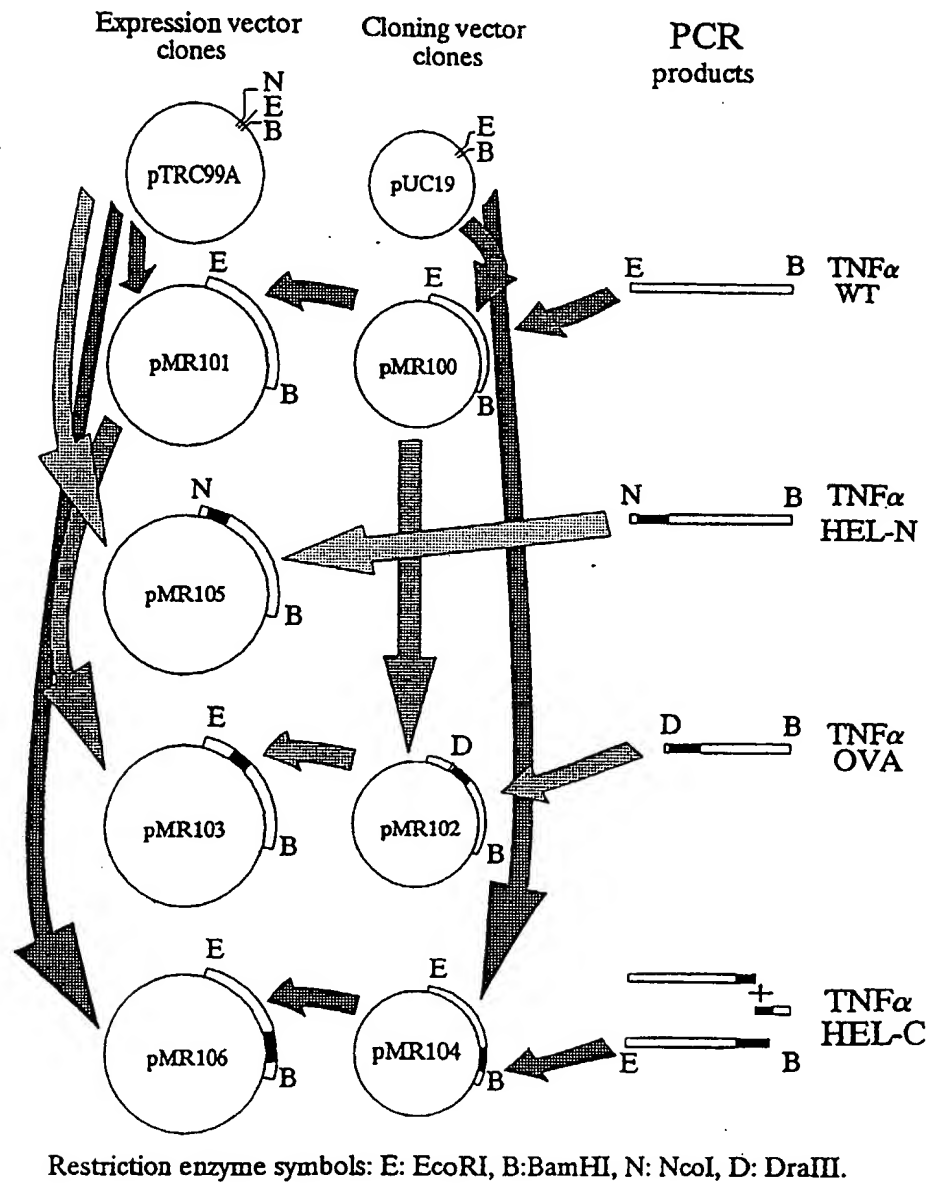


FIG. 3

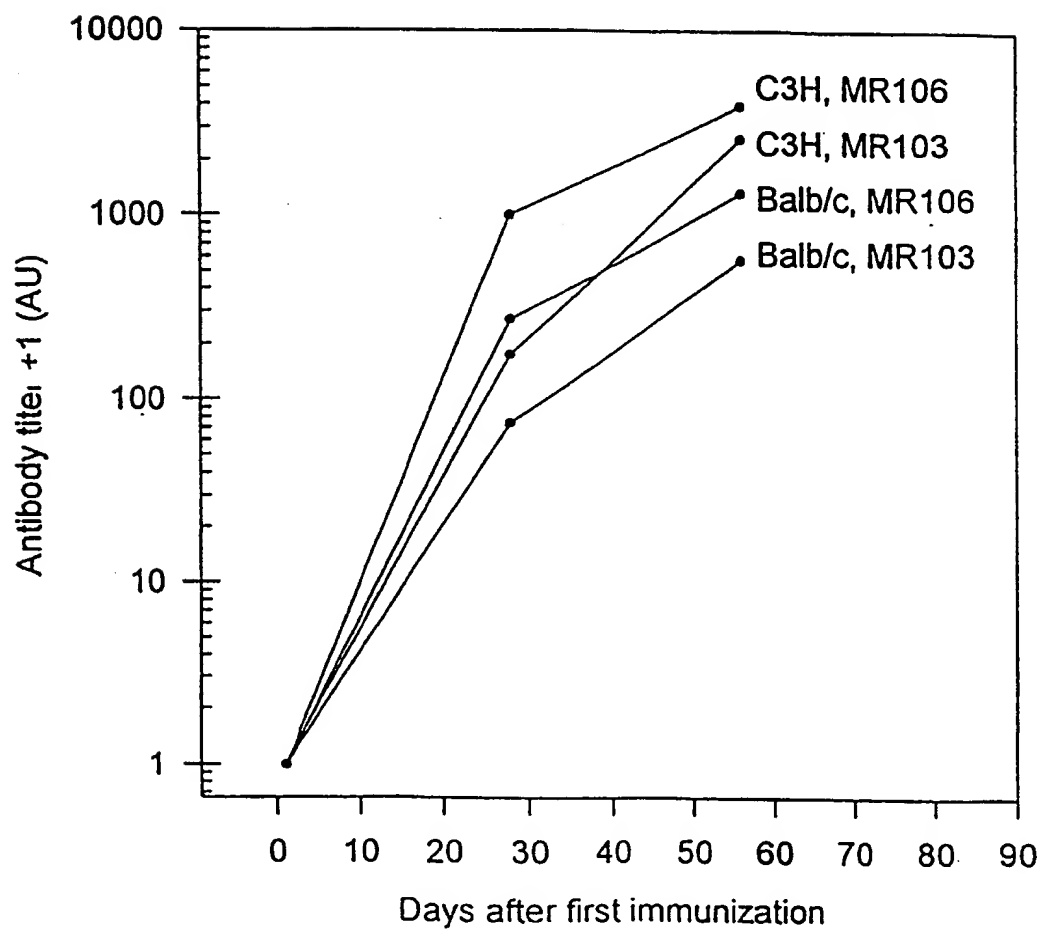
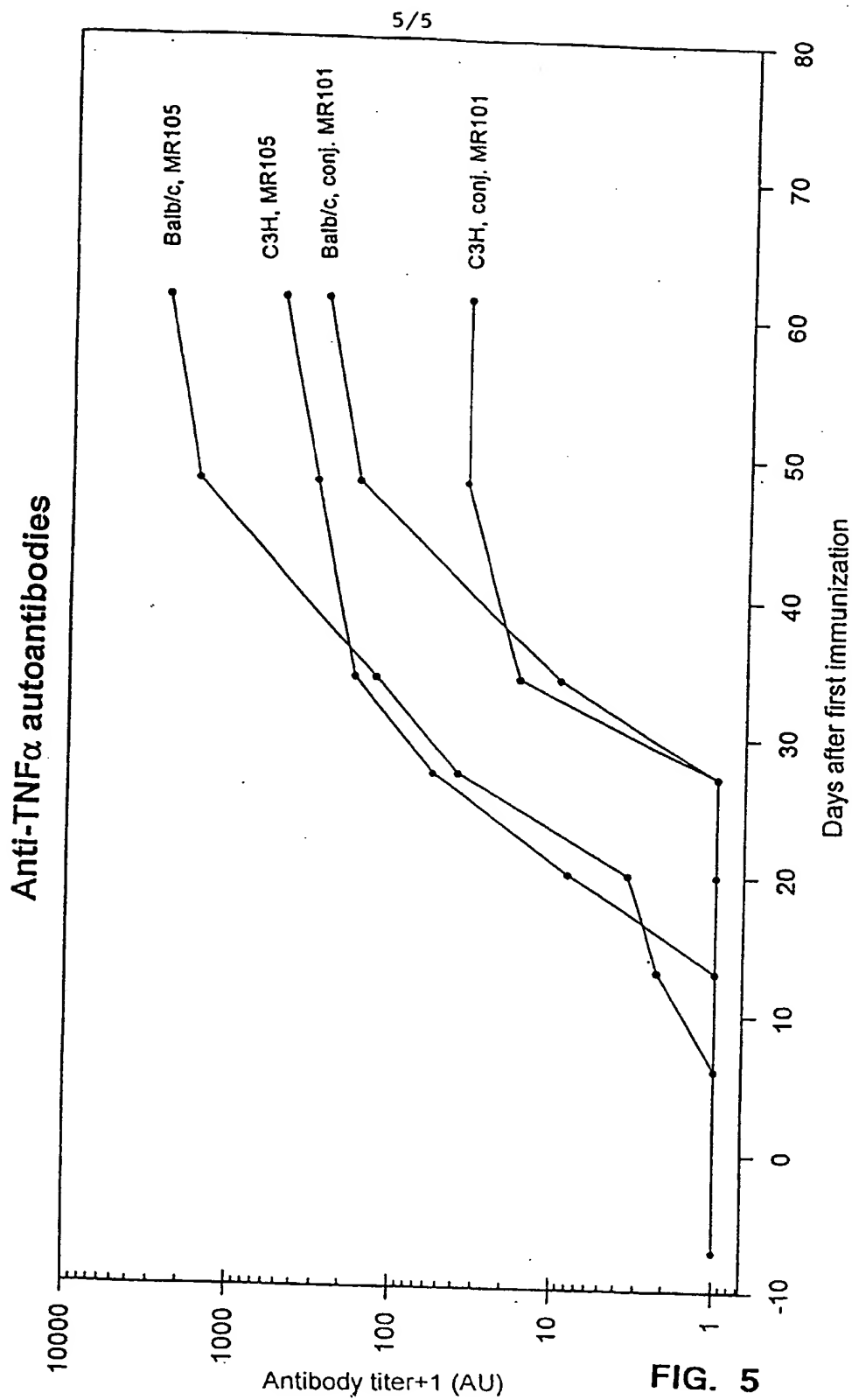
Anti TNF $\alpha$  auto-antibodies

FIG. 4

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